Qualitative Comparison of Cassiicolin in Four Strains of Corynespora cassiicola

V. PUJADE-RENAUD[1], Y. BOURRE[1], F. DE LAMOTTE[1], J. PONCET[3], D. BIEYSSE[4]

Corynespora cassiicola *is a necrotrophic ascomycete fungus affecting a wide range of plants. In rubber tree,* C. cassiicola *causes the Corynespora Leaf Fall (CLF) disease, responsible for sporadic but often severe epidemics in rubber plantations in most Asian and African producing countries. Divergence in the susceptibility of rubber clones to CLFD in various locations revealed the existence of different physiological races.*
A toxin secreted by the fungus (cassiicolin) has been identified as the primary determinant of C. cassiicola *pathogenicity. An optimized purification protocol allowed the preparation of highly purified toxin in sufficient amount to fulfil its molecular characterization. Cassiicolin was shown to be a 27 amino acids glycosylated protein with 3 disulfide bounds.*
To test whether qualitative differences in the toxin may influence the pathogenicity, four strains of various geographic origins and with different virulence profiles were analysed comparatively. Biochemical purification of the toxin followed by mass spectrometry was attempted from all four strains, as well as cloning and expression analysis of the full cassiicolin-encoding gene, when detected. The results are discussed with respect to the pathogenicity of the selected strains.

Key words: Corynespora cassiicola, toxin, cassiicolin, gene

---

[1] UMR DAP (CIRAD and INRA), TA A-96/03, Avenue Agropolis, 34398 Montpellier cedex 5, France.
[3] IGF, CNRS UMR 5203, 141 rue de la Cardonille, F-34094 Montpellier cedex 5, France.
4 UMR BGPI (CIRAD), Campus International de Baillarguet, TA A-54 / K, 34398 Montpellier Cedex 5, France.

Corynespora Leaf Fall Disease (CLFD) is an important disease of rubber tree, caused by the necrotrophic fungus *Corynespora cassiicola*. Since its first report in the late fifties, the pathogen has rapidly spread over most rubber producing countries in Asia and Africa where it causes severe epidemics and yield losses. Emergence of new physiological races of *C. cassiicola* showing preferential host range in rubber plantations has also been reported and a number of studies on the genetic diversity among isolates have been developed (Silva *et al.*, 1995, 1998, 2003, Hashim and Indran 1999; Saha *et al.*, 2000; Atan *et al.*, 2003, Romruensukharom *et al.*, 2005, Nghia *et al.*, 2008). However the determinism of the host range specificity remains unknown.
A secreted toxin, named cassiicolin, was shown to be the primary determinant of the pathogenicity in *C. cassiicola* strain CCP (Breton *et al.*, 2000). An optimized purification protocol allowed the preparation of highly purified toxin in sufficient amount to fulfil its molecular characterization (Barthe *et al*., 2007; de Lamotte *et al*, 2007*)*. Cassiicolin was shown to be a 27 amino acids glycosylated protein with a compact 3-dimensional structure knitted by three disulfide bounds. The sugar moiety, located on the second amino acid was identified as a methyl-mannose. However, no sequence homology could be identified and the mode of action of the toxin remains unknown.
It has been proposed (Breton *et al.*, 2000) that the interaction between *C. cassiicola* and rubber

tree may follow a quantitative model according to which the amount of toxin produced would determine the virulence (specific pathogenicity) of a given strain. This hypothesis was based on the good correlation observed between the toxicity (measured using the leaf wilting assay) of the culture filtrates from four different strains, and the amount of toxin (estimated by ELISA dot-blot) in these filtrates. However, this quantitative model does not exclude the possible occurrence of qualitative variations of the toxins from one strain to another, such as sequence or structure differences, which may also influence the pathogenicity. To test this hypothesis, we selected four strains of various geographical origins with contrasted patterns of virulence for a qualitative comparison of their toxins. Toxins were first purified from the culture filtrates and analyzed by mass spectrometry. In a second step, cloning of the cassiicolin gene from strain CCP provided PCR tools for a thin comparison of the toxins at the primary structure level.

EXPERIMENTAL

Biological material

C. cassiicola strains originate from rubber plantations in Cameroun (strains BCA1 and BCA3), Philippines (strain CCP) and Sri Lanka (Strain Sri15). The mycelium was grown for toxin production in 100 ml of modified Czapeck medium, for 20 days, as previously described (de Lamotte *et al,* 2007*)*. The mycelium was collected by filtration of the culture medium through Whatman paper, deep-frozen in liquid nitrogen and kept at -80°C. The filtrate was filter-sterilized twice through 0.22 µm Millipore membranes, under sterile laminar flow and stored at 4°C. Bioassays were conducted as previously described (de Lamotte *et al,* 2007*)* on the susceptible rubber tree cultivars PB260.

Cassiicolin purification

Cassiicolin purification was conducted as described previously (de Lamotte *et al,* 2007*)*. Briefly, the sterile filtrate was first pasteurized at 45°C to reduce its viscosity and neutralised by adding 1/3 volume of 0.2M K_2HPO_4. It was then submitted to reverse phase chromatography (RPC) using GE-HealthCare Source 15 RPC (1 cm x 8.5 cm) equilibrated with buffer A *(* $KHPO_4$ 10 mM pH 7). Elution was performed by a linear gradient (20 column volumes, 4 $ml.min^{-1}$) from buffer A to buffer B (buffer A with 70% acetonitrile). The toxicity of the eluted fractions was monitored by bioassays on PB260 detached leaves, after slight evaporation of the fractions (to half of the initial volume) in order to eliminate the acetonitrile. The toxic fractions were pooled and concentrated by a second run on GE-HealthCare Source 15 RPC in similar conditions except that the elution was done in 3 column volumes. The toxic fraction was identified by bioassay and submitted to size exclusion chromatography on a GE-Healthcare Superdex 30 Prep-Grade (1.6 cm x 60 cm) equilibrated with buffer C ($KHPO_4$ 10 mM pH 7, acetonitrile 10%, pH7) and eluted at 1 $ml.min^{-1}$. Elution of the toxin was monitored by bioassay, after partial evaporation. A final concentration step was performed on GE-HealthCare Source 15 RPC and the purified toxin was stored at 4°C.

Mass spectrometry

Nanoelectrospray mass spectrometry was performed as described previously (de Lamotte *et al,* 2007*)*, on a Quadrupole Time-Of-Flight (Q-TOF) mass spectrometer (QSTAR Pulsar-*i,* Applied Biosystems, Foster City, CA) fitted with a Protana nanospray inlet system (Protana, Odense,

Denmark). Spectra were recorded using the Analyst QS software (Applied Biosystems). Parameters were adjusted as follows: ion spray voltage (IS), 900 V; curtain gas (CUR), 25; declustering potential (DP), 10–45 V; focusing potential (FP), 265 V; declustering potential 2 (DP2), 15 V. Fragmentation experiments (CID) were performed in the collision cell using nitrogen gas on the doubly or triply charged ions detected, with a collision energy profile optimized individually (20-65 V). Before being placed in the source tip holder, capillaries (Protana, Odense, Denmark) were loaded with the desalted samples according to a described procedure . Briefly, a 2 µl aliquot of chromatrographically-purified cassiicolin was loaded on Poros 20 R2 (Applied Biosystems) packed in a gel-loader pipette tip, washed with 1 % formic acid (2 µl) and eluted with 2 µl of 50:50:1 methanol/water/formic acid.

Cloning of the cassiicolin cDNA and gene.

The cloning strategy is presented in Fig.1. The detailed procedure will be presented in a separate publication (under preparation). Briefly, a cDNA fragment was amplified by RT-PCR using degenerated primers (F1 and R1) designed from the cassiicolin aminoacid sequence (SwissProt accession P84902). Total RNA was extracted from 100 mg of ground lyophilized mycelium using Trizol reagent (Invitrogen), treated with DNAse I (Fermentas) and reverse transcribed using MMLV-Revertaid (Fermentas). PCR amplification was performed using the "taq CORE Kit 25" (Q-BIOgene), for 30 cycles with annealing at 46°C. The PCR products were cloned in the pCR 2.1 vector using the TA Cloning Kit (Invitrogen) and transferred into *E. coli* DH5α cells. After plating and overnight incubation, 12 colonies were picked up for plasmid extraction (Wizard plasmid extraction kit, Promega) and the plasmids inserts were sequenced (GATC-biotech).

The 5' and 3' ends of the cassiicolin cDNA were cloned using the SMART RACE cDNA amplification kit (Clontech), with specific primers designed from the cassiicolin cDNA fragment (F2 and R2). The amplification products were ligated in the pGEMT-easy plasmid then transferred into Electro Max DH10B Phage Resistant bacteria (In vitrogen). Plasmids were extracted from 16 isolated colonies and the inserts were sequenced.

Amplification of the full length cassiicolin cDNA was performed using the primers F3 and R3, designed from the 5' and 3' ends of the RACE-amplified cDNA sequences respectively (Fig. 1). Template was 1µl of reverse-transcribed RNA primed with oligo-dT-NV. A cassiicolin genomic clone was amplified in the same conditions except that the reaction template was 100 ng of genomic DNA.

Blast search analysis was performed using the NCBI tools. Prediction of the signal pepdide was performed using SignalP 3.0 (Bendtsen *et al.*, 2004). Sequence annotation was performed using the VectorNTI suite 7.

Cassiicolin gene detection and comparative RT-PCR analysis in the 4 selected strains

For the detection of the cassiicolin gene in all four selected strains, two sets of primers (F3/R3 and F4/R4) were used. Template was 100 ng of genomic DNA. The F3/ R3 primer set was used to amplify the whole sequence, for 30 cycles with an annealing temperature of 62°C. The F4 and R4 primers, which are framing intron 2, were used to amplify a fragment of the cassiicolin domain, for 30 cycles with annealing at 53°C. A fragment of DNAr (Internal Transcribed Spacers 1 and 2) was amplified using the primers ITS 1 (TCCGTAGGTGAACCTGCGG) and P3 (CCTTGGTCCGTGTTTCAAGACGGG) in order to verify the quality and concentration of the template genomic DNA. Amplification conditions were 30 cycles with an annealing temperature of 55°C.

Comparative RT-PCR analysis was conducted with the primers F5 and R5 (see Fig 1) which amplify the cassiicolin domain and part of the 3'UTR. Template was 1µl of reverse-transcribed RNA primed with oligo-dT-NV. Amplification was performed for 30 cycles with an annealing temperature of 52°C, using the Advantage 2 PCR kit (Clontech).

RESULTS

Cloning of the cassiicolin cDNA and gene

The purification of cassiicolin and the elucidation of its 27 aminoacids sequence (de Lamotte *et al*, 2007; Barthe, 2007 #2129} provided the starting point for the cloning of its cDNA and gene. The cloning strategy is summarized below (Fig1).

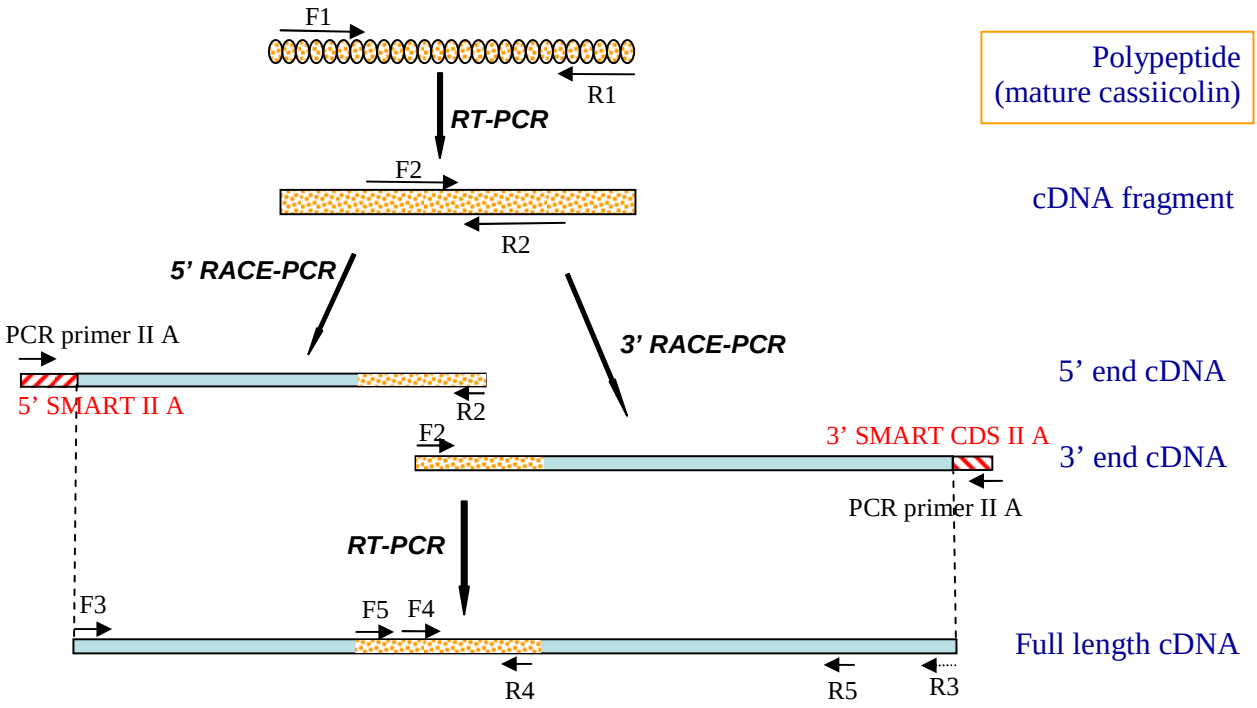

Fig.1: Strategy for the cloning of a full length cDNA encoding cassiicolin

A cassiicolin cDNA fragment was first obtained by RT-PCR amplification using degenerated primers (F1 and R1) designed from the cassiicolin aminoacid sequence, with *C. cassiicola* (strain CCP) total RNA as template. Its deduced amino acids sequence was identical to the sequence of the purified polypeptide, confirming its identity as a cassiicolin-encoding cDNA fragment. Specific primers internal to this cDNA fragment (F2 and R2) were then designed to amplify the 5' and 3' ends of the cassiicolin cDNA. Finally, primers designed from the extremities of the RACE-amplified 5' and 3' fragments (F3 and R3) were used to amplify a 442 bp single chain cDNA. The same terminal primers F3 and R3 were also used to amplify the corresponding genomic sequence (557 bp), using genomic DNA from strain CCP as template.

Fig 2 shows the organization of the cassiicolin precursor gene. The gene contains a 58 amino acids open reading frame organized in two domains. The N-terminal domain encodes a 35 amino acids polypeptide starting with a 17 amino acids putative signal peptide. The C-terminal domain corresponds to the 27 amino acids active cassiicolin. It is interrupted by two introns with typical eukaryotic splice junctions, 5'(-GT) and 3'(-AG). Sequence comparison with public sequence databases failed to reveal any significant homology, whether in nucleic or amino acids.

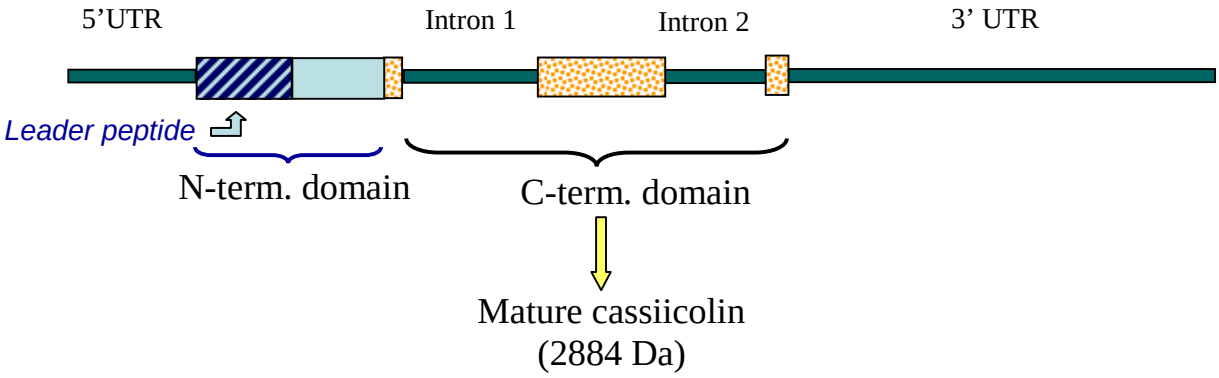

Fig. 2 Genomic organization of the cassiicolin precursor gene

Comparison of four selected *C. casiicola* strains

Four strains of various geographical origins were selected among a collection of *C. cassiicola* isolates from rubber tree, based on their pathogenic profiles. BCA1, BCA3 and CCP were previously shown to be virulent, with very low, medium and high pathogenicity respectively, as estimated by the intensity of symptoms (necrosis size) induced by conidia inoculation on detached leaves from eleven rubber tree cultivars (Breton *et al.*, 2000). In addition, the toxicity of their culture filtrate was evaluated through bioassays on detached leaves from the susceptible cultivar PB260. The necrosis extents induced by the culture filtrate or by fungal inoculation were found in good correlation. The filtrate from strain Sri15 showed no toxicity at all on PB260 and the strain was selected as non virulent.
These four strains were compared for their production of toxin, through biochemical and molecular analyses.

Biochemical purification

Biochemical purification of the toxins from the culture filtrates of the four selected strains was attempted following the previously published protocol. The chromatography profiles, whether "reverse phase" or "size exclusion", were highly reproducible over several runs made from one culture filtrate. However, they diverged completely from one fungal strain to another, indicating that for each new strain, the toxin purification process requires adaptations, with monitoring of

the toxicity through bioassays at each step.
For the CCP strain, 9 ml of pure concentrated active product were obtained out of 780 ml initial sterile filtrate treated in several runs. For the BCA3 strain, 5 ml of pure concentrated active product were obtained out of the 750 ml initial sterile filtrate. For BCA1, the toxicity of the crude filtrate was very low compared to CCP or BCA3. Nevertheless, some toxic fractions could be detected after reverse phase chromatography. They were pooled (54 ml) and concentrated (9ml). However, no toxic fraction could be detected after size exclusion chromatography and the purification process could not be completed. For Sri15, the crude filtrate was submitted to reverse phase chromatography although it did not show any toxicity on PB260 leaves. However, none of the eluted fractions did show any toxicity, even after a 3 time concentration of the fractions by evaporation.

Comparative analysis by mass spectrometry

Mass spectrometry by ESI-QTOF revealed that both CCP and BCA3 purified samples had the same molecular masse (2884.96 Da) indicating that the fungal strains CCP and BCA3 most certainly produced identical toxins, including identical post-translational modifications. Unfortunately it was not possible to perform NMR analysis (with the objective to verify that 3-D structure was also identical in both strains) because the quantity of purified toxin obtained from BCA3 was two low.

Detection and sequence comparison of the cassiicolin gene

Two sets of primers designed from the CCP cassiicolin precursor genomic sequence were used for detecting the cassiicolin gene in all four selected strains. One set (F4 and R4) framing intron 2 amplified a band of identical size (128 pb) in both CCP and BCA3 but failed to amplify any band in BCA1 and Sri15 (Fig. 3). The other set (F3 and R3), previously used for cloning the full cDNA and corresponding gene, amplified the expected 557 bp product in CCP and BCA3 only. A control gene fragment (DNAr Internal Transcribed Spacers 1 and 2 of DNAr) was equally amplified, demonstrating that the genomic DNA used as template was of equal quality and quantity in all four samples.
Cloning and sequencing of the 557 bp PCR product from BCA3 demonstrated that the CCP and BCA3 cassiicolin precursor genes have fully identical nucleotide sequences.

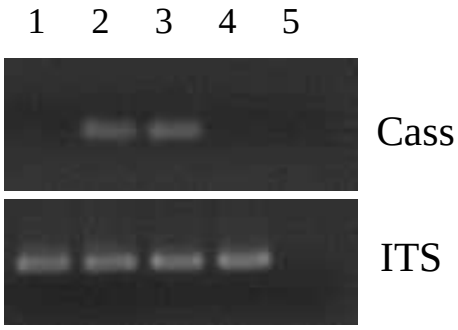

Fig. 3: Detection of the cassiicolin gene in 4 different C. cassiicola *isolates:*
Lane 1, BCA1; lane 2, BCA3; lane 3, CCP; lane 4, Sri15; lane 5, negative control (no DNA template). Genomic DNA (100 ng) was amplified with cassiicolin-specific primers F3 and R3 (upper panel) or control primers ITS1 and P3 (lower panel) corresponding to the DNAr Internal Transcribed Spacers 1 and 2.

Cassiicolin gene expression was analyzed comparatively in the mycelium of all four strains collected after 20 days of culture, which was the date chosen for optimum mycelium development and toxin production. Bands of the expected size were amplified for BCA3 and CCP (Fig. 4). However, the BCA3 band was of lower intensity compared to CCP suggesting a lower level of expression for the cassiicolin gene precursor from BCA3 compared to CCP under the *in vitro* culture conditions applied.

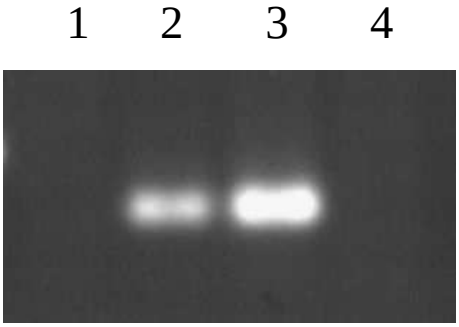

Fig. 4: RT-PCR amplification of a cassiicolin cDNA fragment :
Lane 1, BCA1; lane 2, BCA3; lane 3, CCP; lane 4, Sri15. First strain cDNA (1µl), synthesized from 5µg DNAse-treated total RNA, was amplified with cassiicolin-specific primers F5 and R5, for 30 cycles, at 52°C annealing temperature.

DISCUSSION

We have previously purified cassiicolin, the toxin responsible for the specific pathogenicity of *Corynespora cassiicola* (strain CCP), and demonstrated that it is a small glycoprotein of 27 aminoacids (de Lamotte *et al*, 2007). In the present work, we demonstrate that cassiicolin originates from a precursor protein synthesized as a direct gene product. Such situation is rather unusual in the fungal kingdom were most host-selective toxins (HSTs) are secondary metabolites of diverse chemical structure, synthesized through complex assembling processes involving multiple enzymes encoded by clusters of co-regulated genes (Wolpert et al. 2002).
Cassiicolin precursor is 58 amino acids protein composed of a 17 amino acids signal peptide which may potentially be necessary for the secretion of the toxin outside the fungus cell, a 14 amino acids domain which may act as facilitator during the excretion process, and the 27 amino acids C-terminal domain corresponding to the mature toxin. Such structure reminds the genomic organization of ToxA, a host-selective toxin from the wheat pathogen *Pyrenophora-tritici-repentis*, the first protein HST identified among fungi, which is also matured from a precursor protein (Ciufetti *et al*., 1997). The cassiicolin precursor gene lacks sequence homology, whether at the nucleic or amino acids level, confirming the novelty of this molecule.

Our biochemical and molecular comparative analysis of toxin production in four strains (CCP, BCA3, BCA1 and Sri15) of different geographical origins and with contrasted patterns of

virulence (highly virulent, moderately virulent, slightly virulent and non-virulent respectively) revealed a rather complex situation.

The absence of toxicity of Sri15 on the susceptible clone PB260 can logically be explained by the absence of cassiicolin gene. On the other hand, the strains showing moderate and high toxicity (BCA3 and CCP) both carry the cassiicolin gene in their genome. This gene is expressed in the mycelium under *in vitro* culture conditions and toxin is produced. We demonstrated that toxins from both strains were identical, first in their primary structure, as the cassiicolin precursor gene sequences were strictly identical, but also most certainly in their post-translational modifications considering that their molecular masses were strictly identical. Therefore, the difference in toxicity/virulence between the two strains could not be attributed to differences in sequence or structure. It seems correlated instead to the level of gene expression which conditions the level of toxin production. The quantitative model is therefore coherent for these 3 clones. Nevertheless, we demonstrated (in case of Sri15) that the absence of virulence is not due to down regulation but rather to the absence of the cassiicolin gene.

The case of BCA1 is atypical in that no cassiicolin gene was detected in that strain, although a slight toxicity was observed for its crude culture filtrate as well as for some of the fractions collected after reverse phase chromatography. The reason why the purification process could not be completed for BCA1 may be that the toxic compound was present in such a low amount that it became too diluted and undetectable over the successive purification steps. Another reason may be that the purification process may appear unsuitable if the BCA1 toxic molecule differs from cassiicolin in its structure or chemical nature. Cassiicolin gene detection in this strain failed, although different set of primers were tested, whether located in the mature cassiicolin domain or in the UTR. This supports the hypothesis that the toxic molecule produced by BCA1 is significantly different from cassiicolin.

In conclusion, this analysis provides first elements indicating that *C. cassiicola* pathogenicity may be rather complex. The molecular tools provided by the cloning of the cassiicolin precursor gene will allow the screening of large collections of *C. cassiicola* isolates. This exploration may lead to the identification of new virulence factors and will help understanding the virulence determinism and the host preferences of the various *C. cassicola* physiological races.

REFERENCES

Atan, S. and Hamid N.R. (2003). Differentiating races of *Corynespora cassiicola* using RAPD and Internal transcribed spacer markers. *J. Rubb. Res.* 6(1): 58-64.

Barthe, P., Pujade-Renaud, V., Breton, F., Gargani, D., Thai, R., Roumestand, C. and De Lamote, F. (2007) Structural analysis of cassiicolin, a host-selective protein toxin from *Corynespora cassiicola*. *J. mol. Biol*. 367, 89-101.

Bendtsen, J. D., Nielsen, H., von Heijne, G. and Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 340(4) : 783-95.

Breton, F., Sanier, C. and d'Auzac, J. (2000). Role of cassiicolin, a host-selective toxin, in pathogenicity of *Corynespora cassiicola*, causal agent of a leaf fall disease of *Hevea*. *J. Rubb. Res.* 3(2):115-128.

Ciuffetti, L. M, Tuori, R. P. and Gaventa, J. M. (1997). A single gene encodes a selective toxin causal to the development of tan spot of wheat. Plant Cell 9(2): 135-44

De Lamotte, F., Duviau, M.-P., Sanier, C., Thai, R., Poncet, J., Bieysse, D., Breton, F., and Pujade-Renaud, V., (2006). Purification and characterisation of cassiicolin, the toxin produced by *Corynespora cassiicola*, causal agent of the leaf fall disease of rubber tree. *J. Chromatogr. B.* 849(1-2): 357-362.

Ismail Hashim and Jeyanayagi Indran (1999) Occurrence and Identification of Physiological Races of *Corynespora cassiicola* of *Hevea*, Proceeding of IRRDB Symposium 1999, Hainan Publishing House, p. 263-272.

Nghia, N. A., Kadir, J., Sunderasan, E., Puad Abdullah, M., Malik, A. and Napis, S. (2008) Morphological and Inter Simple Sequence Repeat (ISSR) Markers Analyses of Corynespora cassiicola Isolates from Rubber Plantations in Malaysia. Mycopathologia

Romruensukharom P., S. Tragoonrung, A. Vanavichit and T. Toojinda (2005) Genetic Variability of *Corynespora cassiicola* Population in Thailand. J. Rubb. Res., 8(1), 38-49.

Saha, T., Arun Kumar, A.S. Sreena, Annakutty Joseph, C. Kuruvilla Jacob, R. Kothandaraman and M.A. Nazeer (2000) Genetic variability of *Corynespora cassiicola* infecting *Hevea brasiliensis* isolated from the traditional rubber growing areas in India, Indian Journal of Natural Rubber Research, 13 (1&2):1-10.

Silva, W.P.K.; Multani, D.S.; Deverall, B.J.; Lyon, B.R. (1995) RFLP and RAPD analyses in the identification and differentiation of isolates of the leaf spot fungus *Corynespora cassiicola*; Australian Journal of Botany [Aust. J. Bot.], vol. 43, no. 6, pp. 609-618

Silva, W.P.K; Deverall, B.J.; Lyon, B.R. (1998) Molecular, physiological and pathological characterization of *Corynespora* leaf spot fungi from rubber plantations in Sri Lanka, Plant Pathology, 47 (3) pp. 267-277

Silva, W.P.K.; Karunanayake, E.H.; Wijesundera, R.L.C; Priyanka, U.M.S. (2003), Genetic variation in *Corynespora cassiicola*: A possible relationship between host origin and virulence, Mycological Research [Mycol. Res.]. Vol. 107, no. 5, pp. 567-571.

Wolpert, T. J., Dunkle, L. D., Ciuffetti, L. M.(2002) Host-selective toxins and avirulence determinants: what's in a name? Ann. Rev. Phytopathol. 40: 251-85